EXHIBIT 1

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Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice

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ABSTRACT **Cells from transgenic mice expressing a human mini-gene for collagen I were used as markers to follow the fate of mesenchymal precursor cells from marrow that were partially enriched by adherence to plastic, expanded in culture, and then injected into irradiated mice. Sensitive PCR assays for the marker collagen I gene indicated that few of the donor cells were present in the recipient mice after 1 week, but 1–5 months later, the donor cells accounted for 1.5–12% of the cells in bone, cartilage, and lung in addition to marrow and spleen. A PCR *in situ* assay on lung indicated that the donor cells diffusely populated the parenchyma, and reverse transcription–PCR assays indicated that the marker collagen I gene was expressed in a tissue-specific manner. The results, therefore, demonstrated that mesenchymal precursor cells from marrow that are expanded in culture can serve as long-lasting precursors for mesenchymal cells in bone, cartilage, and lung. They suggest that cells may be particularly attractive targets for gene therapy *ex vivo*.**

Many recent efforts at gene therapy have pursued the strategy of isolating hematopoietic stem cells from bone marrow, genetically altering the cells *ex vivo*, and then returning the cells to patients (see refs. 1–5). In addition to hematopoietic stem cells, marrow contains mesenchymal precursor cells (6) that produce fibrous tissue, bone, or cartilage when implanted into appropriate tissues *in vivo* (7–10) and that generate colonies of fibroblastic (6, 11–17), adipocytic (14), and osteogenic (14) cells when cultured under appropriate conditions. One of the most distinctive features of mesenchymal cells that synthesize bone, cartilage, and other connective tissues is their high level of expression of genes for specific types of collagen (see ref. 18). Here we have used cells from a transgenic mouse line that expresses a human mini-gene for collagen I in a tissue-specific manner (19–21) to see whether precursor mesenchymal cells from marrow that are expanded in culture can serve as long-term precursors of bone and other connective tissues after intravenous infusion into irradiated mice.

MATERIALS AND METHODS

Preparation of Donor Cells. Cultured adherent cells from marrow were prepared from transgenic mice expressing a marker human *COL1A1* mini-gene (19–21). Freshly isolated nonadherent cells from marrow were prepared from normal mice of the same inbred FVB/N strain. Tibias and femurs were dissected from 8- to 10-week-old mice, the ends of the bones were cut, and marrow was flushed out with 2 ml of ice-cold α-modified minimum essential medium (α-MEM; Sigma) containing 10% (vol/vol) fetal bovine serum (FBS) by using a needle and syringe. The pooled marrow cells were dispersed by agitation in the syringe and the cells were counted electronically by using a Coulter model ZM counter. From 5×10^6 to 5×10^7 nucleated bone marrow cells in 25 ml of α-MEM 10% FBS were plated onto 75-cm² culture flasks. After 4 h, nonadherent cells were removed by replacing the medium. The adherent cells were expanded from 5×10^4 to 1×10^7 cells by culture for 7–10 days with replacement of medium on day 7. The cells were recovered by digestion with 0.25% trypsin for 20 min.

Radiation and Transplantation. Recipients were 8- to 10-week-old normal FVB/N mice. Before cell infusions, half the mice were irradiated with a ^{137}Cs irradiator (Atomic Energy, Ottawa). The unit had a dose rate of 116 cGy/min with a parallel opposed beam configuration. Each animal received 9.0 Gy in two 4.5-Gy sessions with a 4-h interval between (22). One to 2 h after the second irradiation, a mixture of 1×10^5 cultured adherent cells from the transgenic mice and 6×10^5 freshly isolated nonadherent cells from normal mice in 0.3 ml of α-MEM/10% FBS was injected into a tail vein of each recipient.

DNA Assays. Freshly dissected tissue was quick frozen in liquid nitrogen. The tissue was then wrapped in aluminum foil and crushed with a hammer. A small aliquot of the crushed tissue was taken for DNA assays, and the rest of the tissue was used to extract RNA. For DNA isolation, the tissue was incubated at 55°C overnight in 0.2 ml of 10 mM Tris·HCl, pH 8.0/50 mM KCl/5 mM $MgCl_2$ gelatin (0.5 mg/ml)/1% Brij 35/proteinase K (0.4 mg/ml). The DNA was denatured at 100°C for 8 min and immediately cooled on ice. PCR primers were designed to amplify simultaneously both the marker mini-gene for human type I procollagen (*COL1A1*) and the endogenous *COL1A1* gene (referred to hereafter as the mouse *COL1A1* gene) and to produce products of different length that were easily distinguishable by PAGE (21). A two-primer assay was developed to assay samples with a low ratio of the marker gene to the endogenous gene and a three-primer assay was developed to assay higher ratios (Fig. 1). In both assays, the 5′ primer (BS49, CAGTCGTCGGAGCAGACGGGAGTTT) hybridized to a sequence common to exon 1 of both human and endogenous mouse *COL1A1* genes. The primer was end-labeled with [^{32}P]dATP by using the T4 polynucleotide kinase terminus-labeling system (GIBCO/BRL). For the two-primer assay, the 3′ primer (BS47, ACTCCCCAGAGTTTGGAACTTACTGTC) was targeted to a downstream sequence of exon 1 in the human sequence but the same primer also amplified a homologous region in the mouse *COL1A1* gene. Because of a natural deletion in the mouse sequence, the product from the human gene was 285 bp and the product from the mouse gene was 225 bp. In the three-primer assay, a second 3′ primer (BS48, ACTCCCAAAAGTTTGGGACTTACTGTC) was targeted to mouse sequence and the product from the mouse gene was again 225 bp. The conditions for PCR were 1 min at 94°C and 2 min at 67°C for 31 cycles. Ten microliters of the reaction products was denatured at 100°C for 8 min and electrophoresed on a 7% polyacrylamide gel containing 6 M

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Abbreviations: FBS, fetal bovine serum; RT, reverse transcription.
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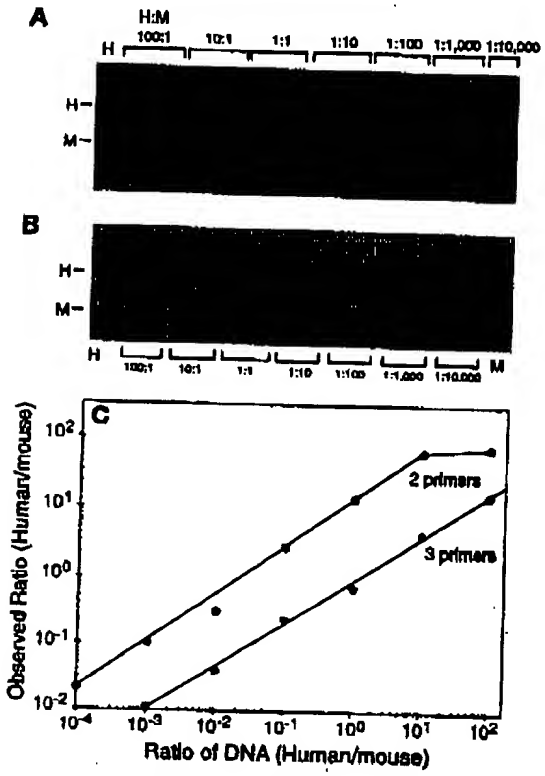

FIG. 1. PCR assays of mouse *COL1A1* gene (M) and marker gene of human mini-*COL1A1* gene (H). Standards were obtained by assays on 50 ng of samples containing the mixtures of mouse and human DNA indicated. The gels were assayed with a phosphostimulable storage plate (PhosphorImager; Molecular Dynamics). (*A*) Two-primer PCR assay for detection of human *COL1A1* gene (H) and endogenous mouse *COL1A1* gene (M). (*B*) Three-primer PCR assay of same mixtures of DNA. (*C*) Observed ratios of human and mouse *COL1A1* genes as a function of ratios of human and mouse DNAs in mixtures assayed. The response of this phosphostimulable storage plate was linear over a 10^7-fold range, but the print-out of the imager had a more narrow range. Therefore, the less intense bands are not visible in *A* and *B*.

urea. The gel was fixed, dried, and assayed by exposing the gel to a phosphostimulable storage plate (Molecular Dynamics model 400S PhosphorImager).

RNA Assays. Total cellular RNA was isolated from tissues by extraction with acidic guanidinium thiocyanate/phenol/chloroform and precipitation with isopropanol (23). One to 5 μg of total RNA was reverse-transcribed in a 20-μl reaction mixture by using 100 pmol of primer (BS84, ACAGCACTCGCC) that was specific for the 3′ ends of both the human and mouse mRNAs and a reagent kit for first-strand cDNA synthesis (Superscript, GIBCO/BRL). The cDNA was amplified in a 50-μl reaction mixture by using each primer at 50 pM and a PCR reagent kit (GeneAmp, Perkin–Elmer/Cetus). A two-primer assay was used to assay expression of the marker human mini-*COL1A1* gene and a three-primer assay was used to assay expression of both genes. For both assays, the 5′ primer (BS83, CTCCGGCTCCTGCTCCTCTTA) was targeted to coding sequences in exon 1 of both the human and mouse *COL1A1* genes. It was 5′ labeled with [^{32}P]dATP. For the two-primer assay, the 3′ primer (BS84, GGACAGCACTCGCCCTCGG) was targeted to coding sequences in exon 2 of the human *COL1A1* gene. For the three-primer assay, the second 3′ primer (BS81, GCACAGACATCGCCCTCCC) was targeted to sequences in exon 2 of the mouse *COL1A1* gene. Conditions for PCR were 1 min at 94°C and 2 min at 60°C for 25 cycles in a 50-μl reaction mixture. The products were separated by gel electrophoresis as described above for DNA and the gels were analyzed either by autoradiography or with a phosphostimulable storage plate.

Assay of Tissue Sections by PCR *in Situ*. Tissues were fixed in 4% (vol/vol) formaldehyde and paraffin sections of ≈3 μm were prepared. The sections were digested with proteinase K (6 μg/ml) for 45 min at 55°C (24). The marker *COL1A1* mini-gene was amplified by PCR using primers (BS66, AGCGGAAGGCGCGATATAGAGTATC, and BS68, CTCCTCCCCCTCTCCATTCCAACT) targeted to exon 1 of the human *COL1A1* gene. PCR conditions were 1 min at 94°C and 2 min at 67°C for 31 cycles in a reaction mixture containing 25 mM dGTP, 25 mM dCTP, 25 mM dATP, 15 mM dTPP, and 10 mM digoxigenin-dUTP (Dig-11-UTP; Boehringer Mannheim). The products were visualized with fluorescein-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim) and UV microscopy.

Isolation of Bone and Cartilage Cells. To isolate bone cells, tibias and femurs were dissected from mice, the ends of the bones were cut, and the marrow was flushed out three times with 2 ml of ice-cold α-MEM/10% FBS (24). Eight of the bones from one mouse were crushed in a sterile mortar and pestle in 3 ml of phosphate-buffered saline (PBS). One milliliter of collagenase (Boehringer Mannheim; 1 mg/ml) in PBS was added and the samples were incubated at 37°C for 5 min. Five milliliters of α-MEM/15% FBS was added, and large fragments were removed by passing the samples through a large-pore wire mesh. Cells in the filtrate were diluted to 25 ml with α-MEM/10% FBS and plated in 75-cm^2 flasks. The cells were cultured for 1 week and then recovered by digestion with 0.25% trypsin for 20 min.

To isolate chondrocytes, xiphoid and articular cartilages were removed from the mice, adherent tissues were removed under a dissecting microscope, and the cartilage was minced under a dissecting microscope. The samples were incubated with hyaluronidase (Worthington; 0.5 mg/ml) in PBS at 37°C for 5–10 min, washed, and incubated with trypsin (2 mg/ml) and bacterial collagenase (2 mg/ml) (Worthington) in PBS at 37°C for 1 h to remove adherent tissues. Washed cartilage fragments were then digested by incubation with collagenase (0.5 mg/ml) in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C for 18 h. The isolated chondrocytes were recovered by centrifugation and washed with DMEM/10% FBS.

RESULTS

Assays for the Marker Gene in Donor Cells. The marker gene consisted of an internally deleted mini-gene for the human proα1 (I) chain of procollagen I that caused synthesis of shortened proα1 (I) chains (19–21). Cells expressing the gene were obtained from a line of transgenic mice (line 73) in which the copy number of the human mini-gene relative to the endogenous mouse gene was ≈100:1, and the steady-state levels of mRNA from the human mini-gene relative to mRNA from the endogenous mouse gene was ≈0.5:1 in most tissues (19–21). Donor cells from marrow partially enriched for mesenchymal precursors were prepared by using protocols (6–17) in which marrow cells that tightly adhere to plastic were isolated and grown in culture in the presence of FBS but in the absence of growth factors that favor the replication of hematopoietic precursors. As reported (6, 11, 12), foci containing two to four fibroblast-like cells appeared in 2–3 days, and the foci grew rapidly into colonies over 7–10 days. By phase-contrast microscopy (data not shown), most of the cells were fibroblast-like, but a few macrophages, endothelial cells, and adipocytes were also seen.

To follow the fate of the donor cells, two PCR assays that simultaneously amplified both the human *COL1A1* mini-gene and the mouse endogenous *COL1A1* gene were developed. With a two-primer assay, the values for the ratio of the human genes to mouse genes were linear over a range of 10^{-4} to ≈10^{+1}

Table 1. Repopulation of tissue by adherent marrow cells intravenously administered to irradiated mice

Tissue	Donor cells, % of total											
	Day 1			Day 7			Day 30			Day 150		
	A	B	C	A	B	C	A	B	C	A	B	C
Marrow	0.006	0.01	0.4	0.07	0.6		7.0	5.0	6.5	5.0	5.0	1.0
Spleen	0.02	0.008	0.1	0.1		0.1	8.0	2.0	12.0	5.5	6.0	5.5
Bone	0.002	0.0001	0.001	0.0005	0.04	0.4	9.0	10.5	4.0	5.5	4.0	2.0
Lung	0.001	<0.0001	<0.0001	0.002	1.0	1.0	3.0	5.0	8.0	2.0	3.5	4.0
Cartilage										3.0	5.0	1.5
Brain			<0.0001	0.005	0.005	0.0005	0.02	0.02	0.02	0.1	0.6	0.2

Donor cells were assayed with the two PCR assays shown in Fig. 1. Because the copy number of human mini-gene in the donor cells was about 100:1, the observed values of ratios of the two DNAs were corrected by a factor of 100. Three mice killed on each day were arbitrarily labeled A, B, and C. Combined values for marrow, spleen, bone, and lung for day 30 did not show a statistically significant difference from the combined values for day 150 (6.8 ± 3.0% and 4.1 ± 6.7%, respectively, mean ± SD; $n = 12$).

(Fig. 1). With the three-primer assay, the values were linear over a range of $\sim 10^{-3}$ to 10^{+2}. Since the donor cells contained ~100 copies of the marker mini-gene, the two-primer assay was linear for detection of 10^{-6} to 10^{-1} donor cell per recipient cell. The three-primer assay was linear for detection of 10^{-5} to 1 donor cell per recipient cell.

Assays of Donor Cells in Recipient Mice. Normal mice were injected with a 1:6 mixture of cultured adherent cells containing the marker mini-gene of *COL1A1* and nonadherent cells from normal mice. DNA assays of recipient mice that had received lethal irradiation indicated that after 1 day only trace amounts of the donor cells were present in marrow, spleen, bone, lung, or brain (Table 1). Slightly higher levels were seen at 7 days. At 30 days and 150 days, progeny of the donor cells accounted for 2.0–12% of the cells in marrow, spleen, bone, and lung (Table 1 and Fig. 2). At 150 days, they also accounted for 1.5–5.0% of the cells in xiphoid cartilage that was dissected free of any mineralized or fibrous tissue under a microscope. Although the mean values appeared to show a decrease between 1 and 5 months (Fig. 2), there was no statistically significant decrease in the combined values for marrow, spleen, bone, and lung between these two time periods (Table 1). Assays of nonirradiated mice revealed only very low levels of the donor cells at the same time points (<0.0001–0.05%).

To confirm that progeny of the donor cells were present in cartilage, chondrocytes were isolated from xiphoid and articular cartilage by microscopic dissection followed by digestion with bacterial collagenase. PCR assays indicated that progeny of the donor cells accounted for 2.5% of the isolated chondrocytes (data not shown).

Location of the Donor Cells in Lung. To determine whether progeny of the donor cells were diffusely incorporated into tissues, tissue sections were assayed by a PCR *in situ* assay in which both primers were specific for the human marker, and one primer was fluorescently labeled (25). Tissue sections of lung demonstrated that progeny of the donor cells were evenly distributed in the parenchyma of both alveoli and bronchi (Fig. 3). Similar assays on sections of bone and cartilage did not provide convincing data because of high background staining and other artifacts (data not shown).

Assays for Expression of the Marker Collagen Gene as RNA. To determine whether the donor cells became functional mesenchymal cells in the tissues they populated, tissues from the recipient mice were assayed by reverse transcription (RT)–PCR for expression of the marker *COL1A1* mini-gene contained in the donor cells. In three mice assayed at 150 days, the mini-gene was expressed in bone (Fig. 4), a tissue in which >50% of the protein synthesized is collagen I (see ref. 18 and references therein).

To confirm expression of the marker *COL1A1* gene in bone, tibias and femurs were dissected from mice, and bone cells were isolated. The cells were grown in culture for 1 week and then assayed with a three-primer RT–PCR assay that detected products from both the marker *COL1A1* mini-gene and the endogenous mouse *COL1A1* gene. The results demonstrated the presence of mRNA from both the marker gene and endogenous gene (Fig. 4).

Expression of the mini-gene for collagen I was more variable in marrow, spleen, and lung (Fig. 5), tissues in which the rate of collagen I synthesis is less than in bone (see ref. 18). As expected, the mini-gene was not expressed in cartilage, a tissue in which ~70% the protein synthesized is collagen II but in which there is no synthesis of collagen I (see ref. 18). To confirm the lack of expression of the marker *COL1A1* mini-gene in cartilage, chondrocytes were isolated from xiphoid and articular cartilage. Assays of the isolated chondrocytes with both a two-primer and a three-primer RT–PCR assay did not detect transcripts from the marker *COL1A1* mini-gene (data not shown).

DISCUSSION

Bone marrow cells that tightly adhere to plastic have been studied extensively (11–17) since Friedenstein *et al.* (6) dem-

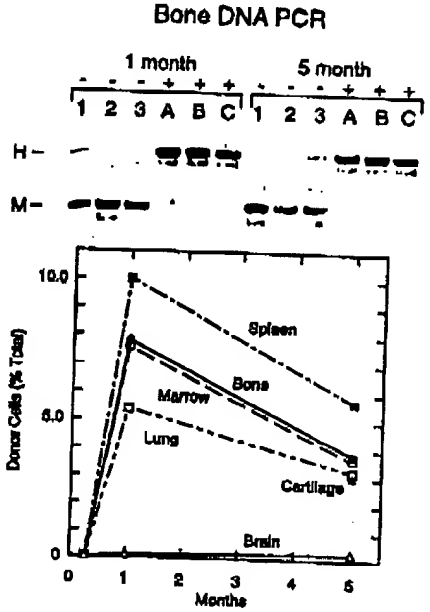

FIG. 2. Percent of donor cells found in tissues of recipient irradiated mice. (*Upper*) Two-primer assay on bone from three mice not irradiated before injection of donor cells (lanes 1–3) and three mice irradiated before the injection (lanes A–C). Bands from the mouse *COL1A1* gene in samples A–C were detected in images printed at higher sensitivity and with the three-primer assay on the same samples (data not shown). (*Lower*) Mean values from Table 1.

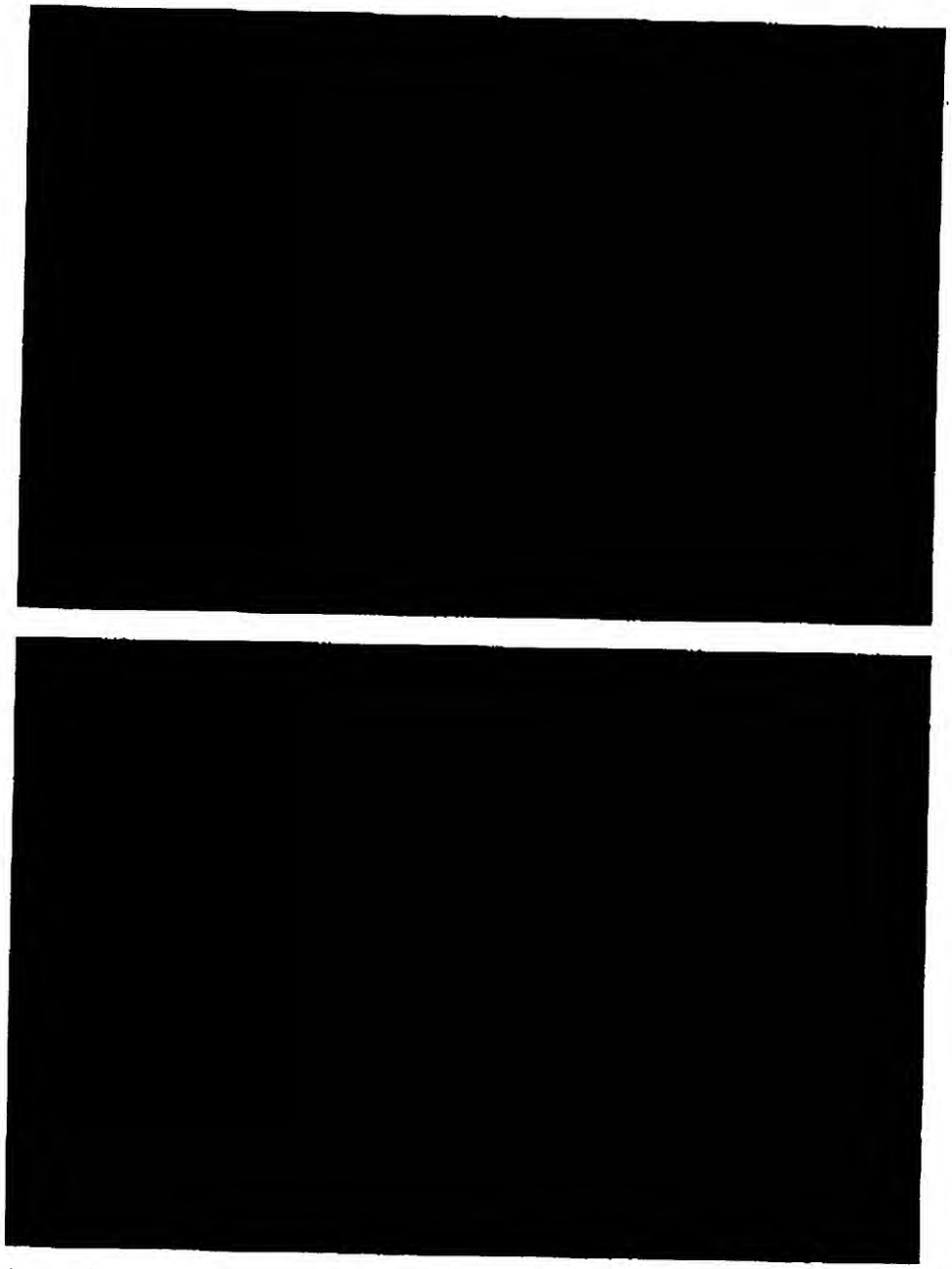

FIG. 3. PCR *in situ* assay (25) for the marker human mini-*COL1A1* gene in sections of lung. (*Upper*) Lung from control mouse. (*Lower*) Lung from irradiated recipient mouse 5 months after injection of donor cells. (×16.)

onstrated that they include a population of fibroblast precursors in addition to hematopoietic precursors. If the adherent cells are cultured in the presence of hydrocortisone or other selective conditions (14–17, 26, 27), populations enriched for hematopoietic precursors or osteogenic cells are obtained. If cultured for ~1 week under the conditions employed here, the predominant cells in the cultures are fibroblast-like (6, 11–17). Assays of the cells with cytochemical markers (11, 12) or for mRNAs (14) indicated that the cells synthesize collagen I, collagen III, fibronectin, alkaline phosphatase, and osteopontin, but most of the cells do not have features characteristic of macrophages, granulocytes, T lymphocytes, B lymphocytes, or endothelial cells. The results here demonstrate that after intravenous injection into irradiated mice, the expanded cultures of adherent cells continue to replicate *in vivo*, and over a period of weeks they populate several connective tissues. The results also demonstrate that the cells serve as long-term precursor cells for these tissues, since they expressed the marker *COL1A1* mini-gene in a tissue-specific manner and were diffusely incorporated into the mesenchymal parenchyma of lung. The wide dissemination of the cells is probably explained by the earlier observations (11, 12) that the marrow cells initially isolated by adherence to plastic are nonreplicating but are activated to replicate by culture *in vitro*. As suggested by Bienzle *et al.* (27), the activation to replication caused by culturing of marrow cells *in vitro* apparently continues after the cells are injected *in vivo*.

Since the donor cells employed here are easily isolated and expanded in culture, they offer an attractive vehicle for gene

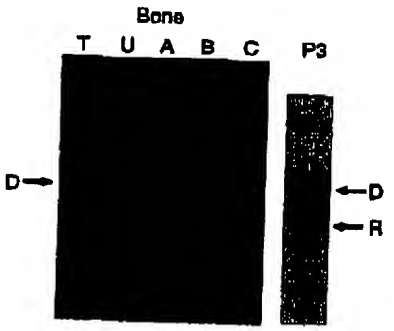

FIG. 4. RT–PCR assays for expression of the marker human *COL1A1* mini-gene in bone. Lanes: T, bone RNA from transgenic mouse with human *COL1A1* mini-gene as control; U, bone RNA from untreated control mouse; A–C, bone RNA from three recipient irradiated mice at 150 days; P3, RNA from 1-week culture of cells from crushed bone from a recipient irradiated mouse killed at 150 days. Bands: D, RT–PCR product of 260 bp from expression of the *COL1A1* mini-gene present in donor cells; R, RT–PCR product of 232 bp from expression of the endogenous mouse *COL1A1* gene. The two-primer RT–PCR assay that detected only the *COL1A1* mini-gene mRNA was used to assay samples of bone and the two-primer assay that detected expression of both genes was used to assay the cultured bone cells. Additional bands are RT–PCR artifacts seen in most reaction products with RNA from control mice.

therapy. Allographic transplants of expanded cultures from matched donors may be effective for therapy of genetic diseases of collagen such as osteogenesis imperfecta and chondrodysplasias in which there is decreased synthesis of normal collagen (see ref. 18). Gene therapy *ex vivo* in which the patient's own cells are manipulated in culture may be effective for the same diseases and for diseases caused by deficiencies of other secreted proteins such as factor VIII and factor IX in hemophilia. X-ray irradiation of recipient mice was necessary in the experiments described here, and the need for marrow ablation will limit application of the strategy. However, Stewart *et al.* (28) have observed that unusually large and repeated administrations of whole marrow cells produced long-term engraftment of hematopoietic precursors into mice that had not undergone marrow ablation. Also, Bienzle *et al.* (27) used marrow cells that were activated by long-term culture to

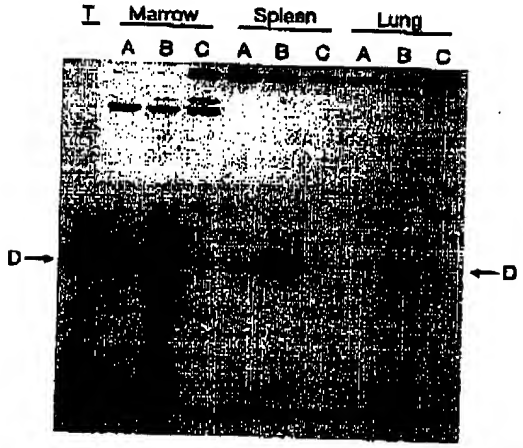

FIG. 5. RT–PCR assays of marrow, spleen, and lung from irradiated recipient mice at 150 days. Conditions, bands, and lanes are as in Fig. 4 for two-primer assay.

permanently populate hematopoietic cells in dogs without marrow ablation. Therefore, large and repeated administrations of cultures enriched for mesenchyme precursor cells may make it possible to use the cells to effectively populate many connective tissues without the need for x-ray irradiation or other measures to suppress immunohematopoietic cells in the recipient.

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